Effect of chronic administration of an aromatase inhibitor to adult male rats on pituitary and testicular function and fertility

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Abstract

The aim of the present study was to evaluate the effects of the administration of a potent non-steroidal aromatase inhibitor, anastrozole, on male reproductive function in adult rats. As anastrozole was to be administered via the drinking water, a preliminary study was undertaken in female rats and showed that this route of administration was effective in causing a major decrease in uterine weight ($P<0{\cdot}02$). In an initial study in male adult rats, anastrozole (100 mg/l or 400 mg/l) was administered via the drinking water for a period of 9 weeks. Treatment with either dose resulted in a significant increase ($\sim$10%) in testis weight and increase in plasma FSH concentrations ($P<0{\cdot}01$) throughout the 9 weeks. Mating was altered in both groups of anastrozole-treated rats, as they failed to produce copulatory plugs. Histological evaluation of the testes from anastrozole-treated rats revealed that spermatogenesis was grossly normal. In a more detailed study, adult rats were treated with 200 mg/l anastrozole via the drinking water for periods ranging from 2 weeks to 1 year. Plasma FSH and testosterone concentrations were increased significantly ($P<0{\cdot}001$) during the first 19 weeks of treatment. However, LH concentrations were increased only at 19 weeks ($P<0{\cdot}001$) in anastrozole-treated rats, and this coincided with a further increase in circulating and intratesticular testosterone concentrations ($P<0{\cdot}05$). No consistent change in inhibin-B concentrations was observed during the study. Suppression of plasma oestradiol concentrations could not be demonstrated in anastrozole-treated animals, but oestradiol concentrations in testicular interstitial fluid were reduced by 18% ($P<0{\cdot}01$). Mating was again inhibited by anastrozole treatment, but could be restored by s.c. injection of oestrogen, enabling demonstration that rats treated for 10 weeks or 9 months were still fertile. Testis weight was increased by 19% and 6% after treatment for 19 weeks and 1 year, respectively. Body weight was significantly decreased ($P<0{\cdot}01$) by 19 weeks of anastrozole treatment; after 1 year the animals appeared to have less fat as indicated by a 27% decrease in the weight of the gonadal fat pad. The majority of anastrozole-treated animals had testes with normal spermatogenesis but, occasionally, seminiferous tubules showed abnormal loss of germ cells or contained only Sertoli cells. Ten percent of anastrozole-treated animals had testes that appeared to contain only Sertoli cells, and one rat had 'giant' testes in which the tubule lumens were severely dilated. Morphometric analysis of the normal testes at 19 weeks showed no difference in the number of Sertoli cells or germ cells, or the percentage volumes of the seminiferous epithelium, tubule lumens and interstitium between control and anastrozole-treated rats. On the basis of the present findings, oestrogen appears to be involved in the regulation of FSH secretion and testosterone production, and is also essential for normal mating behaviour in male rats. Furthermore, these data suggest that the brain and the hypothalamo–pituitary axis are considerably more susceptible than is the testis to the effects of an aromatase inhibitor. Anastrozole treatment has resulted in a model of brain oestrogen insufficiency.

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Introduction

The only roles of oestrogens in adult males were believed to be in the regulation of sexual behaviour and gonadotrophin secretion, but numerous new data have led to increasing interest in other roles of oestrogen in normal male reproductive development and function (reviewed in Sharpe 1998). This has been stimulated by the description of mutations in the genes coding for either oestrogen receptor-α (ERα) or cytochrome P450 aromatase in men or transgenic mice, as some of the data would imply that compromised oestrogen action has a deleterious effect on male fertility (Smith *et al.* 1994, Morishima *et al.* 1995, Eddy *et al.* 1996, Carani *et al.* 1997, Robertson *et al.* 1999).

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In addition, a second oestrogen receptor (ERβ) has been cloned from human testis and rat prostate cDNA libraries (Kuiper *et al.* 1996, 1997, Mosselman *et al.* 1996), and it has been demonstrated that both ERα and ERβ are expressed widely in the testis, male reproductive tract and accessory sex organs throughout development and in adulthood (Fisher *et al.* 1997, Hess *et al.* 1997*b*, Saunders *et al.* 1997, 1998), including the expression of ERβ in pre- and post-meiotic germ cells (Saunders *et al.* 1998).

The production of oestrogen from androgen is catalysed by aromatase cytochrome P450, which is found in many tissues, including the gonads, brain, adipose tissue, bone and heart (Simpson *et al.* 1997, Sharpe 1998). Approximately 20% of oestrogens present in blood are produced by the testis (de Jong *et al.* 1973). Within the rat testis, Leydig cells express aromatase (Tsai-Morris *et al.* 1985), as do immature Sertoli cells (Tsai-Morris *et al.* 1985, Dorrington & Khan 1993). More recently, germ cells and spermatozoa have been shown to possess aromatase activity (Janulis *et al.* 1996, 1998), leading to the suggestion that germ cells may modulate their own environment via oestrogen production, both in the testis and as they pass through the excurrent ducts (Janulis *et al.* 1996, 1998).

The main approach used to explore new roles for oestrogens in the male has centred on targeted inactivation of ERα (ERKO), ERβ (BERKO) and aromatase (ArKO) genes in mice. Studies of ERKO mice suggest that oestrogens are important for normal sperm production (Eddy *et al.* 1996). Although a grossly normal male reproductive tract develops in these mice, spermatogenesis was found to be impaired in adulthood, resulting in the production of fewer sperm with very low fertilising ability, thus resulting in infertility. Further investigation showed that fluid resorption within the efferent ducts was impaired (Hess *et al.* 1997*a*), and that this led to progressive accumulation of fluid and damage to spermatogenesis (Eddy *et al.* 1996). A role for oestrogen in the regulation of fluid resorption by efferent ducts fits with previous data showing that the efferent ducts show the most pronounced expression of ERα in the male reproductive system (Fisher *et al.* 1997, Hess *et al.* 1997*b*). Preliminary reports on the effects of disruption of either ERβ (Krege *et al.* 1998) or aromatase (Fisher *et al.* 1998) genes in male mice indicate that they show major differences in phenotype when compared with the ERKO mice (Eddy *et al.* 1996). Young adult male BERKO mice are fertile and the male reproductive tract appears grossly normal, and the only abnormality that has been reported so far is hyperplasia of the bladder and prostate epithelium in older males (Krege *et al.* 1998). Likewise, young adult ArKO mice are also fertile, although older males show progressive disruption of spermatogenesis, resulting in infertility caused by a defect in spermiogenesis, rather than fluid accumulation (Fisher *et al.* 1998, Robertson *et al.* 1999); apparently, disruption of spermatogenesis occurs much earlier when the mice are fed on a soya-free diet, which contains much lower concentrations of phytoestrogens (Simpson *et al.* 1999).

An alternative, and more 'physiological' approach to identifying the roles of oestrogen in the male is to suppress activity of aromatase in otherwise normal animals via the administration of selective and potent inhibitors. The aim of the present study was therefore to evaluate the effect of long-term treatment with an aromatase inhibitor on blood hormone concentrations, spermatogenesis and fertility in adult male rats. We have used a highly selective and potent non-steroidal aromatase inhibitor, anastrozole 2,2′[5-(1H-1,2,4-triazol-1-ylmethyl)-1,3-phenylene]bis (2-methylpropiononitrile), marketed as Arimidex (Astra–Zeneca), which has been developed for the treatment of postmenopausal women with breast cancer (Plourde *et al.* 1994). Anastrozole is highly specific and is able to inhibit oestrogen synthesis without affecting any of the other enzymes involved in steroid biosynthesis; in addition, it has no intrinsic hormonal activity (Dukes *et al.* 1996). Oral administration of a single dose of 0·1 mg/kg anastrozole to adult female rats on day 2 or 3 of oestrus is sufficient to block ovulation (Dukes *et al.* 1996), whereas daily administration of the same dose to immature female rats inhibited androstenedione-induced uterine hypertrophy (Dukes *et al.* 1996). In male pigtailed monkeys, twice-daily oral administration of 0·1 mg/kg anastrozole for 7 days suppressed plasma oestradiol concentrations by 50% (Dukes *et al.* 1996), whereas administration to healthy men induced a dose-dependent suppression of oestradiol (Plourde *et al.* 1994). The preclinical pharmacology of anastrozole suggested that it might be a useful tool with which to investigate the actions of oestrogens *in vivo*.

Materials and Methods

Animals and treatments

Adult male Wistar rats aged 80–90 days and female immature Wistar rats aged 21 days, bred in the Centre for Reproductive Biology, were used for these studies and were maintained in a controlled environment with free access to food and water. The rats were fed a standard soy-containing diet. Rats were administered a selective aromatase inhibitor, anastrozole, which was kindly provided by Astra-Zeneca Pharmaceuticals (Alderley Park, Cheshire, UK).

Administration of anastrozole to female rats

To demonstrate that the exposure of rats to anastrozole via the drinking water was a suitable method of administering the compound, an initial study was performed that involved the treatment of immature female rats. On day 21 of postnatal life, eight female rats were given a regimen of drinking water containing 200 mg/l

anastrozole, and a group of seven age-matched control rats remained on normal drinking water supply. When the rats reached 50 days of age, they were killed by CO_2 inhalation followed by cervical dislocation. Body weight and the weights of the ovaries and uterus were recorded and peripheral blood was collected for measurement of hormone concentrations.

Administration of anastrozole to adult male rats

Experiment 1 This was a preliminary study that compared the effects of two doses of anastrozole on hormone concentrations, mating behaviour and testicular histology in adult male rats. In this experiment, 42 rats were divided into three groups: a control group of 14 rats that received normal tap water, and two treated groups of 10 and 18 rats respectively, which were administered anastrozole in their drinking water at concentrations of 100 mg/l or 400 mg/l. Blood samples were collected (see below) from all animals before the start of treatment and after 1, 3 or 6 weeks of treatment. After 7 weeks of treatment, 3–11 rats from each group were perfusion fixed with Bouin's (see below) after collection of a final blood sample. The remaining rats were placed with female rats to assess fertility (see below) and then, at 9 weeks, were killed by CO_2 inhalation followed by cervical dislocation. Body and various organ weights were recorded and peripheral blood was collected.

Experiment 2 This was a detailed time-course study of the effects of anastrozole on male reproductive function. A total of 89 rats were used: 34 controls receiving tap water and 55 receiving anastrozole in their drinking water at a concentration of 200 mg/l. This dose was used because results of experiment 1 indicated that there was no obvious difference between the effects seen with 100 or 400 mg/l anastrozole. Groups of control and anastrozole-treated rats were sampled at weeks 2, 10, 19 and after 1 year of treatment (2 weeks: $n=7$ control, $n=13$ treated; 10 weeks: $n=5$ control, $n=24$ treated; 19 weeks: $n=14$ control, $n=11$ treated; 1 year: $n=8$ control, $n=7$ treated). The majority of the rats treated for periods up to 19 weeks were bled before commencement of treatment and subsequently at weeks 1, 3, 6 and 15. No pretreatment blood samples were collected from the rats treated for 1 year, but they were bled after 14 weeks and 6 months of treatment. At the end of the treatment period, blood was collected and tissue weights recorded and some of the rats were perfusion-fixed with Bouin's, so that various tissues could be processed for histological evaluation (see below). At 10 weeks, fertility was evaluated in a subgroup of 10 treated animals, but no blood or tissue samples were collected from these rats. Fertility was also evaluated in all the rats that had received anastrozole treatment for 9 months, after which these rats were replaced on treatment for a further 3 months (see below).

In addition to the groups described above, another group of rats (eight treated and four controls) were administered 200 mg/l anastrozole via their drinking water for 48 h, after which peripheral venous (PV) blood and testicular interstitial fluid were collected (see below) for the measurement of oestradiol concentrations.

Collection of blood samples and testicular interstitial fluid

Samples of PV blood, collected before and during treatment, were obtained by incising a tail vein while rats were anaesthetised with halothane (Astra- Zeneca). Blood was collected into a heparinised tube, plasma separated by centrifugation at 3500 r.p.m. for 30 min at 4 °C and stored at −20 °C for subsequent hormone measurement. At the end of treatment, blood was collected by cardiac puncture using a heparinised syringe and needle after the animals had been killed by CO_2 inhalation and cervical dislocation.

Testicular vein (TV) blood was also collected from the animals in experiment 2, which had been treated for 19 weeks, to determine intratesticular testosterone concentrations (Maddocks & Sharpe 1989). Rats were anaesthetised with halothane and heparinised by injection of 125 IU heparin (Leo Laboratories Ltd, Princes Risborough, Bucks, UK) into the left femoral vein. The right testis was exposed via a scrotal incision and TV blood collected into haematocrit tubes by puncturing the major surface testicular vein with a 28-gauge needle at a point just before its division into the mediastinal venous plexus at the anterior pole of the rete (Maddocks & Sharpe 1989).

Testicular interstitial fluid was collected overnight at 4 °C, essentially as described by Sharpe & Cooper (1983). The rats were killed by inhalation of CO_2 followed by cervical dislocation. The testes were removed and an incision was made at the caudal end of the testicular capsule. The testes were then placed in a plastic tube and the interstitial fluid was allowed to drain from them over the next 16 h. The testes were removed, the tubes centrifuged at 1000 *g* for 5 min and the interstitial fluid aspirated and stored at −20 °C.

Tissue recovery

In experiment 1, the following tissues were removed and weighed: testis, caput epididymis, cauda epididymis, ventral prostate, seminal vesicles, left kidney, adrenals and pituitary gland. In experiment 2, testis and ventral prostate weights were recorded for each rat.

Some of the treated and control rats were anaesthetised with halothane and perfusion-fixed via the thoracic aorta, first with 0·9% saline containing 0·01% heparin until the testicular blood vessels cleared, and then with Bouin's for 40 min, as described previously (Millar *et al.* 1993). Between five and 11 anastrozole-treated rats were perfusion-fixed at each time-point, and a total of 18

control rats representing a spread of time-points; the size and morphology of testes from control rats did not vary detectably at the different sampling time-points. Various tissues were collected, including the testes, rete testis, efferent ducts, caput epididymis, corpus epididymis, cauda epididymis, ventral prostate, seminal vesicles and adrenals. Once the tissues were removed they were weighed then placed into Bouin's for a further 5 h before being transferred to 70% alcohol. Before processing, Bouin's-fixed testicular tissue was cut transversely into at least five slices with a razor blade. The fixed tissue was then processed for 17·5 h in an automated Shandon processor and embedded in paraffin wax.

Mating and fertility studies

In experiment 1, each rat (seven or eight from each group) was withdrawn from treatment after 7 weeks and placed in a mating cage with an adult female for a maximum of 1 week. The cages were checked daily for the presence of copulatory plugs as an indication that mating had occurred. Once a copulatory plug was detected, the rat was placed back on the appropriate treatment for a minimum of 1 week before being killed at the end of the experiment.

It was found in experiment 1 that treatment with anastrozole prevented mating, so a different approach was used in experiment 2 to assess whether the rats were still fertile after chronic treatment with anastrozole. Ten rats that had been treated with anastrozole for 10 weeks, and eight rats that had been on treatment for 9 months were each given a single s.c. injection of 100 μg diethylstilboestrol (DES; Sigma) in corn oil, to override the effect of aromatase inhibition in the brain, as the conversion of testosterone to oestrogen has been shown to be important for normal sexual behaviour (Meisel & Sachs 1994). The rats were withdrawn from anastrozole treatment at the time of injection of DES and placed in mating cages with an adult female rat for a maximum of 1 week. When a copulatory plug was detected, the female was replaced with another female rat. Mated females were monitored to check whether they became pregnant and allowed to go to term so that litter size and sex ratio could be recorded.

Histology and morphometric analysis of testicular tissue

Tissue sections were cut at 5 μm and floated onto coated slides (2% 3-aminopropyltriethoxy-silane; Sigma) and dried at 50 °C before being stained with haematoxylin and eosin or periodic-acid Schiff's reagent and haematoxylin. The testicular morphology of all animals that were perfusion-fixed was examined. All seminiferous tubules within at least two complete testicular cross-sections per animal were evaluated for any signs of impairment to spermatogenesis. The rete testis and the efferent ducts from each animal were also examined for any evidence of fluid accumulation.

The purpose of the morphometric analysis was to determine, firstly, the efficiency of spermatogenesis by enumerating total germ cell volume supported by each Sertoli cell and, secondly, to assess whether there was any indication of fluid accumulation within the seminiferous tubule lumen or the interstitium after anastrozole treatment for 19 weeks. Cross-sections of testes from four control and five treated rats were examined under oil immersion using a Leitz laborlux microscope fitted with a Leitz 63 × plan apo objective and a 121-point eyepiece graticule. Using a systematic clock-face sampling pattern from a random starting point, 32 fields were counted from a total of three cross-sections per animal. Each section was cut from a different paraffin block, to ensure that different regions of the testis were sampled. Points falling over Sertoli cell or germ cell nuclei, seminiferous epithelium, interstitium or seminiferous tubule lumens were scored and expressed as a percentage of the 121 points possible. For each animal, a mean percentage volume was calculated for each parameter for the 32 fields evaluated. Spermatogonia, spermatocytes and round and elongate spermatids were distinguished, but their total volumes were combined for analysis in these studies. The values for percentage volume were converted to absolute volumes per testis by reference to testis volume (=weight), as shrinkage was minimal. For each animal, the total germ cell volume per testis was expressed relative to total Sertoli cell volume per testis.

Hormone measurements

Plasma follicle-stimulating hormone (FSH) concentrations were determined by RIA using a kit provided by NIDDK (Bethesda, MD, USA) and results expressed in terms of the rFSH-RP-2 standard. Plasma concentrations of inhibin-B were measured using a two-site ELISA, as previously described (Illingworth *et al.* 1996). The assay has been validated previously for measurement of inhibin-B in the rat (Sharpe *et al.* 1999).

Concentrations of testosterone in peripheral and testicular venous plasma were also measured, using an ELISA adapted from an earlier RIA method (Corker & Davidson 1981). Plasma, to which was added trace amounts of ^{3}H-testosterone (Amersham Radiochemicals, Little Chalfont, Bucks, UK), was extracted twice with 10 volumes of hexane : ether (4 : 1, v/v) and the organic phase dried down at 55 °C under N_2. The efficiencies of extraction were 75% and 70% for PV and TV plasma, respectively. The testosterone was measured by ELISA as follows. The second antibody was immobilised to the ELISA plate by the addition of 100 μl acid-purified donkey anti-goat/sheep IgG (250–350 μg/ml), diluted in 0·1 M sodium carbonate buffer, pH 9·6. The plate was sealed and incubated overnight at 4 °C. The wells were washed twice with 0·1% Tween-20 and then blocked for 10 min at room temperature with 200 μl of the same solution. Samples were assayed in 50 μl duplicates, diluted

Table 1 Effect of treatment of immature female rats from days 21 to 50 of postnatal life, with 200 mg/l anastrozole added to drinking water on organ weights and hormone concentrations (means ± S.E.M.)

	Uterus weight (mg)	Ovary weight (mg)	FSH (ng/ml)	Testosterone (ng/ml)	Oestradiol (pg/ml)
Treatment					
Control (n=7)	342 ± 54	114 ± 5	5·6 ± 2	0·25 ± 0·05	53·4 ± 4
Anastrozole (n=8)	193 ± 11†	129 ± 4*	7·8 ± 0·8	1·22 ± 0·31**	44·2 ± 2*

*P<0·05, †P<0·02, **P<0·01, compared with control.

in 0·1 M PBS pH 7·4 containing 0·1% gelatin (Sigma) to correspond to a testosterone standard curve ranging from 1 to 500 pg/50 µl. Samples were incubated overnight at 4 °C with 50 µl sheep anti-testosterone-3-cmo BSA (1 : 100 000) and 50 µl testosterone-3-cmo labelled with horseradish peroxidase (1 : 20 000). The following day, the plate was washed several times with 0·1% Tween-20. Then 200 µl substrate solution (5 mM O-phenylenediamine, Sigma) and 0·03% hydrogen peroxidase diluted in 0·1 M citrate/phosphate pH 5·0 were added to each well and the plate incubated in the dark for approximately 10–30 min until the colour reaction was optimal. The reaction was stopped by the addition of 50 µl 2 M sulphuric acid to each well and optical density was read at 492 nm on a plate reader. The limit of detection of the assay was ~12 pg/ml.

Oestradiol concentrations were determined using an oestradiol MAIA RIA kit (Bio-Stat Diagnostic Systems, Stockport, Cheshire, UK). The limit of detection of this assay is ~5 pg/ml and cross-reaction with testosterone is reported to be around 0·0033%. In the present studies, it was confirmed that rat plasma diluted in parallel with the oestradiol standard, and that physiological concentrations of testosterone did not interfere in the assay. Plasma, to which was added trace amounts of ^{3}H-oestradiol (Amersham Radiochemicals), was extracted twice with 10 volumes of ether then the organic phase was dried down at 55 °C under N_2. The efficiencies of extraction were 82% and 72% for PV plasma and interstitial fluid, respectively. Samples were reconstituted in 0·05 M PBS pH 7·4 containing 0·1% gelatin. Samples were assayed in 200 µl duplicates against an oestradiol standard curve ranging from 0·06 to 16 pg/200 µl. Samples were incubated with 100 µl primary antibody (1 : 10) and 100 µl tracer, corresponding to ~12000 c.p.m., at room temperature for 4 h. Then 100 µl normal rabbit serum (1 : 600 dilution) and 100 µl anti-rabbit serum (1 : 30) was added to the samples and incubated overnight at 4 °C. The next day, 1 ml wash solution (containing 0·9% saline v/v, 0·2% triton v/v and 4% polyethylene glycol w/v; Sigma) was added and the tubes centrifuged at 3500 r.p.m. for 30 min at 4 °C. The supernatant was discarded and the pellets were counted after they had been allowed to dry for 30 min. Assay results were interpreted using Assay Zap software (Biosoft, Cambridge, UK).

Statistical analysis

In experiment 1, comparisons of body weight, organ weights and hormone concentrations for the three groups were by analysis of variance and, where significant differences between groups were indicated, sub-group comparisons were by Student's t-test. In experiment 2, any statistical difference between body weight, organ weight and hormone concentrations of the two groups was determined using Student's t-test. The hormone data were also analysed by a second method. The majority of the animals used in these studies were bled before treatment was started, allowing a baseline determination of hormone concentrations. In some instances, pretreatment hormone concentrations were found to be significantly different between control and treated groups. Therefore, hormone concentrations at the various time-points during experiment were also compared with the concentrations present in the pretreatment blood samples for each individual group, using the paired t-test. Mating frequency in control and anastrozole-treated rats was compared using the χ^2 test. Morphometry data were compared using Student's t-test.

Results

Efficacy of the route of administration

Addition of 200 mg/l anastrozole to the drinking water of female rats from postnatal day 21 to day 50 induced a significant reduction in uterine weight, an increase in ovarian weight, a significant increase in peripheral blood concentrations of testosterone and a modest decrease in blood concentrations of oestradiol (Table 1). Plasma FSH concentrations were greater in the anastrozole-treated female rats, but values were not significantly different from those in controls (Table 1).

Experiment 1

Organ weights Analysis of organ weights in male rats that had been exposed to 100 or 400 mg/l anastrozole showed that the only significant difference from controls was in the weights of the testis and ventral prostate: testis weight was significantly increased, whereas ventral

prostate weight was decreased in both anastrozole-treated groups (Table 2). The weights of the epididymis, seminal vesicles, adrenals and pituitary were not altered by anastrozole treatment. Body weight was unaffected by treatment, indicating that the two doses of anastrozole administered were not inducing any general toxic effects on the animals.

Hormone concentrations Plasma FSH concentrations in rats administered 100 mg/l or 400 mg/l anastrozole were increased significantly ($P<0·001$) at most time-points after treatment, compared with baseline pretreatment values (data not shown). The change in FSH concentrations was similar to that described for experiment 2 (see below).

Mating Rats from each of the three groups were mated to assess whether exposure to anastrozole impaired fertility. Of the eight control rats placed individually in mating cages with a female, only one male failed to produce a copulatory plug. All mated females became pregnant, but litter size was not recorded. In contrast, none of seven and one of seven males, respectively, from the 100 mg/l and 400 mg/l anastrozole-treated groups mated – a difference from control that was significant ($P<0·05$) for both groups. Comparison of the control group with the treated groups pooled together gave a statistical significance of $P<0·001$. This inhibition meant that fertility *per se* could not be evaluated in this experiment.

Effect on spermatogenesis and the efferent ducts Histological evaluation of the testes from animals that were perfusion-fixed after treatment with anastrozole for 7 weeks failed to reveal any consistent adverse changes. Spermatogenesis appeared grossly normal, although it appeared that seminiferous tubule lumen size was slightly increased, and that Leydig cell numbers might be reduced in some of the anastrozole-treated animals (not shown). The efferent ducts were also examined; again, some, but not all, of the anastrozole-treated animals were found to have ducts with distended lumens (not shown). Animals exhibiting such changes were equally evident in the 100 mg/l and 400 mg/l anastrozole-treated groups.

In view of the overall findings in Experiment 1, it was decided that a more detailed experiment with a longer duration of treatment and using an intermediate dose (200 mg/l) of anastrozole should be undertaken.

Experiment 2

Effect of chronic administration of 200 mg/l anastrozole on body weight and on the weights of the testis, ventral prostate and gonadal fat pad Rats sampled after 2 weeks of anastrozole treatment showed no change in body weight or in testis weight (Table 3). By 10 weeks of treatment, testis weight had increased marginally ($P<0·05$), whereas body weight remained unaltered (Table 3). However, by 19 weeks, body weight was significantly decreased ($P<0·01$) and testis weight was markedly increased ($P<0·001$), with the exception of two rats with testes that contained Sertoli cells only (SCO), when compared with control values (Table 2). After 1 year of treatment, whereas the control group had continued to gain weight, the anastrozole-treated rats showed a highly significant loss in weight ($P<0·0001$; Table 2). One possible explanation is that the anastrozole was affecting appetite, as the rats showed no other signs of ill health. Testis weight was significantly increased ($P<0·02$), but to a smaller extent than was seen at 19 weeks. At 1 year, the gonadal fat pad was also weighed, because of information indicating that aromatase knockout mice showed increased fat accumulation (Fisher *et al.* 1998). The weight of the gonadal fat pad was reduced in the anastrozole-treated rats ($P<0·02$). Ventral prostate weight was generally reduced in the anastrozole-treated rats, but this did not reach significance at any of the sampling times.

Effect of administration of 200 mg/l anastrozole for 19 weeks on hormone concentrations Blood FSH concentrations were increased relative to controls at all time-points, including the pretreatment values; this can probably be explained by the fact that the control pretreatment FSH concentrations were significantly lower than those measured for subsequent bleeds. Comparison of FSH concentrations within the anastrozole-group relative to the pretreatment values showed that FSH concentration was significantly increased at all time-points ($P<0·001$, Fig. 1). No consistent change in inhibin-B concentrations was observed with treatment (data not shown).

Plasma luteinizing hormone (LH) concentrations were generally similar between control and anastrozole-treated groups (Fig. 1). The only significant differences occurred in the pretreatment and 1-week bleeds. It is not known why the control group had exceptionally high LH concentrations in the pre-bleed samples. Statistical analysis of the LH data from the anastrozole group indicated that LH concentration was increased compared with pretreatment concentrations at week 19 ($P<0·001$).

Blood concentrations of testosterone were increased significantly in anastrozole-treated rats at weeks 1, 2, 6, 15 and 19 compared with control values (Fig. 1). Plasma testosterone concentrations were significantly increased ($P<0·001$) above pretreatment values in the anastrozole-treated rats at the same time-points. Testosterone was increased by ~50% at weeks 1, 3 and 6 of treatment, and by 300% at week 15 and 350% at week 19 relative to pretreatment values. Although testosterone concentrations in controls became significantly increased ($P<0·01$) compared with pretreatment values at weeks 15 and 19, this change was only modest compared with that in the anastrozole-treated animals (Fig. 1). Measurement of testosterone concentrations in testicular venous blood,

Table 2 Effect of treatment of adult male rats for 9 weeks with two doses of anastrozole added to drinking water on body weights and organ weights (means ± S.E.M.)

	Body weight (g)	Organ weight (mg)						
		Testis	Ventral prostate	Seminal vesicle	Kidney	Adrenal	Pituitary	Caput epididymis	Cauda epididymis
Treatment									
Control (n=6)	574 ± 17	1910 ± 34	432 ± 29	341 ± 30	1667 ± 76	58 ± 4	11 ± 0·4	215 ± 4	261 ± 9
Anastrozole									
100 mg/l (n=7)	612 ± 22	2121 ± 58†	415 ± 17	337 ± 25	1675 ± 53	55 ± 3	10 ± 0·3	226 ± 8	285 ± 16
400 mg/l (n=7)	579 ± 11	2060 ± 49*	347 ± 19*	368 ± 20	1709 ± 67	53 ± 2	11 ± 0·2	221 ± 13	282 ± 14

*P<0·05, †P<0·02, compared with control.

Table 3 Effect of chronic treatment with 200 mg/l anastrozole added to drinking water on body, testis, ventral prostate and fat pad weights (means ± S.E.M.)

			Organ weight		
	Treatment	Body weight (g)	Testis (mg)	Ventral prostate (mg)	Fat pad (g)
Treatment duration (weeks)					
2	Control (n=7)	492 ± 16	1934 ± 46	570 ± 33	
	Anastrozole (n=13)	486 ± 11	1926 ± 34	580 ± 27	
10	Control (n=5)	613 ± 16	1986 ± 76	634 ± 29	
	Anastrozole (n=11–14)	594 ± 10	2141 ± 37	544 ± 79	
19	Control (n=14)	641 ± 11	2071 ± 165	689 ± 58	
	Anastrozole (n=11)	580 ± 12**	2464 ± 71***[a]	594 ± 50	
1 year	Control (n=8)	695 ± 16	2011 ± 47	692 ± 39	4·4 ± 0·38
	Anastrozole (n=7)	562 ± 11***	2127 ± 47†	659 ± 57	3·2 ± 0·16†

†P<0·02, **P<0·01, ***P<0·001, compared with respective control value.
[a]Sertoli cell-only testes were excluded from this mean (see Results).

collected from rats sampled at week 19, demonstrated that intratesticular testosterone concentrations were also significantly increased (P<0·05) in the anastrozole-treated rats compared with the controls (Fig. 2).

Plasma oestradiol concentrations were determined for the rats treated for 1, 2 and 19 weeks and for the corresponding control groups, but were not found to be reduced by anastrozole treatment at any of the time points (Table 4). In order to evaluate whether there was any reduction in intratesticular oestradiol concentrations, another group of rats was treated for 48 h with 200 mg/l anastrozole. Peripheral blood concentrations of oestradiol remained unaffected by treatment in these rats, but an 18% decrease (P<0·01) was detected in oestradiol concentrations in interstitial fluid from the treated rats (Fig. 3).

Effect of administration of 200 mg/l anastrozole for 1 year on hormone concentrations Plasma FSH and testosterone concentrations were measured at different times in rats treated with anastrozole for up to 1 year (Fig. 4). Confirming our previous findings, a significant increase in FSH concentrations was observed at 14 weeks relative to controls (P<0·01). However, although FSH concentrations remained higher than in controls for the remainder of anastrozole treatment, this modest increase did not reach significance at 6 months and 1 year. Testosterone concentrations did not differ significantly at any of the time-points (Fig. 4).

Effect of anastrozole on fertility In order to assess fertility of the rats treated for 10 weeks with 200 mg/l anastrozole, 10 rats were each administered a single injection of DES and then placed with a female. This treatment resulted in the production of seven copulatory plugs out of a possible 12 mating opportunities. The seven females became pregnant and gave birth after a normal duration of gestation. Average litter size was found to be normal for our colony of rats (mean ± S.D. 11 ± 3) with a male : female offspring ratio of 6 : 5. Fertility was also evaluated for eight rats that had been treated with 200 mg/l anastrozole for 9 months. After a single injection of DES, three of the rats produced a copulatory plug when placed with a female; the rats became pregnant and gave birth to litters with an average size of 11·7 ± 1·5 (mean ± S.D.) with a male : female offspring ratio of 6 : 6. It was concluded that prolonged treatment of adult rats with 200 mg/l anastrozole does not compromise testicular function.

Effect on spermatogenesis and the efferent ducts Examination of testicular histology at all of the sampling times revealed that spermatogenesis was grossly normal in the majority of the animals (not shown). The only indication of a slight adverse effect of treatment was the increased frequency of occasional seminiferous tubules exhibiting variable degrees of germ cell loss, to the extent that some tubules contained only Sertoli cells. Approximately 1–2% tubule cross-sections were affected in the testes of rats treated for 10 or 19 weeks or 1 year. In addition, a total of five rats from experiment 2 that had been treated with anastrozole for 2, 19 weeks or 1 year had SCO testes. The affected rats were not associated with a particular duration of treatment, but these changes were never observed in the control group. Furthermore, a single anastrozole-treated rat had testes showing pronounced and widespread dilatation of the seminiferous tubule lumens.

At 19 weeks, testis weight in the anastrozole-treated rats (with the exception of the rats with SCO testes) was increased by ~20% in comparison with age-matched controls. Two possibilities that might explain the increase in weight are, firstly, the presence of a greater number of germ cells, suggestive of an increase in the efficiency of spermatogenesis or, secondly, the accumulation of fluid within the interstitial or luminal compartments of the

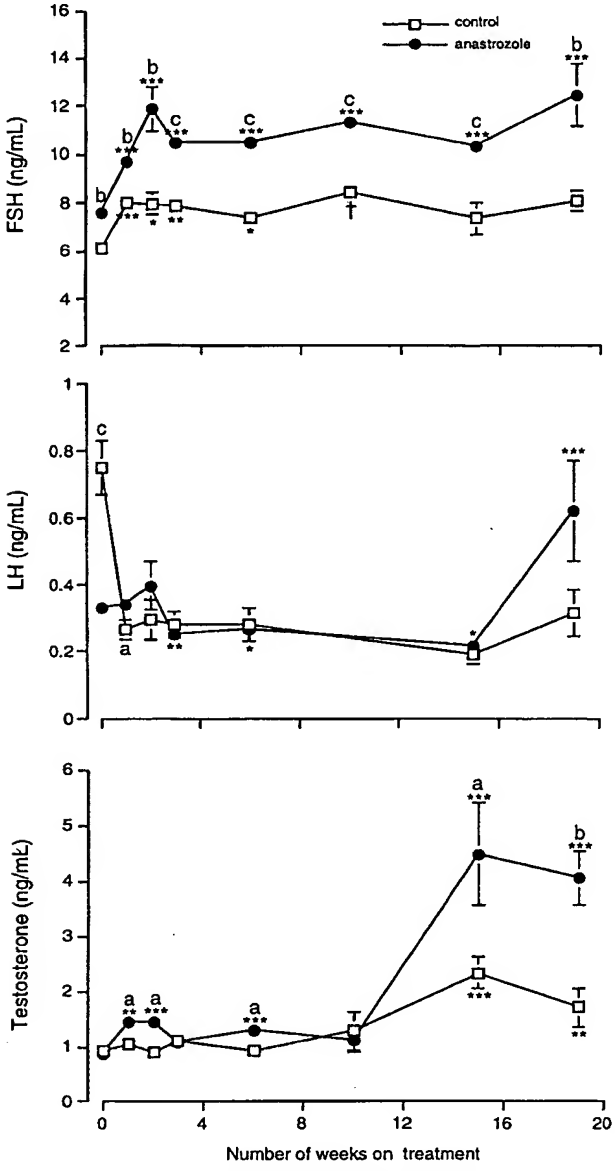

Figure 1 Effect of chronic treatment of adult male rats with 200 mg/l anastrozole in drinking water on mean (± S.E.M.) plasma FSH (top), LH (middle) and testosterone (bottom) concentrations. Control group, $n=5$–21; anastrozole group, $n=7$–40. *$P<0.05$, †$P<0.02$, **$P<0.01$, ***$P<0.001$ compared with respective pretreatment values. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ compared with control group. Note that LH concentrations at 10 weeks have been omitted because insufficient plasma was available to be assayed.

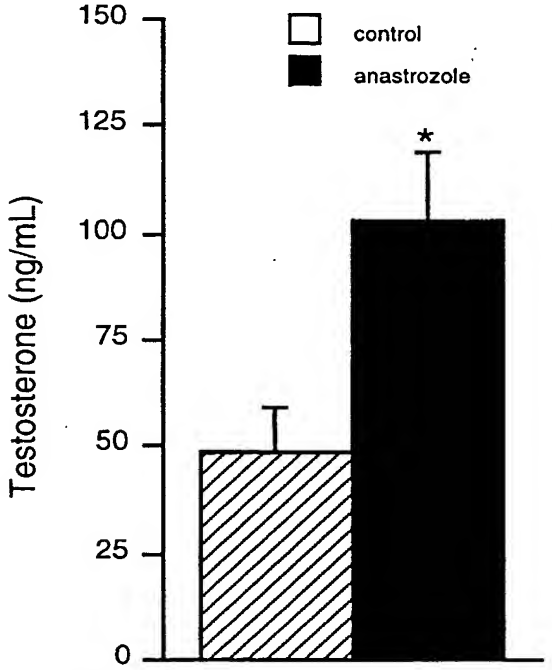

Figure 2 Effect of treatment of adult rats with 200 mg/l anastrozole in drinking water for 19 weeks on concentrations of testosterone in testicular vein blood (mean ± S.E.M., $n=6$–7). *$P<0.05$ compared with the control group.

testis. In order to investigate this, detailed morphometry was performed on paraffin-fixed testis sections. Morphometric analysis showed that the total volume of germ cells relative to the volume of Sertoli cells remained unaltered by anastrozole treatment (Table 5). In addition, the percentage volume of various testicular compartments, including the lumen, interstitium and seminiferous epithelium, were within the control range. The rete testis area was examined in the testes of all the animals. After 19 weeks of treatment, the rete area was distended only in the SCO syndrome and fluid-filled testes. There was no obvious distension in the rete testis of the other treated rats. After 1 year of treatment, there was no evidence of fluid accumulation in the rete testis. The data therefore do not explain why testis weight was increased by anastrozole treatment.

The histology of the efferent ducts of animals treated with anastrozole for 19 weeks or 1 year were also

Table 4 Effect of 200 mg/l anastrozole added to drinking water of adult male rats on oestradiol concentrations in plasma (means ± S.E.M.) after different treatment durations

	Oestradiol concentration (pg/ml)		
	1 week ($n=21$–39)	2 weeks ($n=9$–16)	19 weeks ($n=8$–10)
Treatment group			
Control	31·9 ± 1·3	27·1 ± 1·6	34·4 ± 3·6
Anastrozole	33·4 ± 1·0	30·8 ± 2·1	37·9 ± 2·5

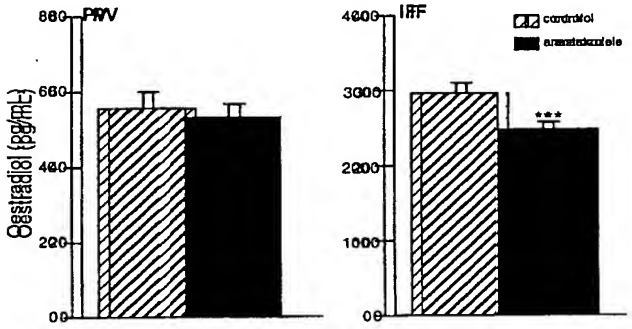

Figure 3 Oestradiol concentrations in peripheral vein blood (PV) and testicular interstitial fluid (IF) in adult male rats treated for 48 h with 200 mg/l anastrozole added to the drinking water (mean ± S.E.M., $n=4–8$). $^{**}P<0·01$ compared with control group.

examined, but no consistent effects were observed with regards to distension of the lumens or reduction in the height of the efferent duct epithelium (data not shown).

Discussion

The aim of these studies was to evaluate the effect, on pituitary and testicular function, of chronic administration of a potent aromatase inhibitor, anastrozole, to adult male rats. The most important effects were on FSH secretion, testosterone production and mating. Plasma FSH concentrations were significantly increased within 1 week of anastrozole treatment, and this was maintained for the first 5 months of treatment; plasma testosterone concentrations were generally increased during the same period. Measurement of intratesticular testosterone concentration after 5 months of anastrozole-treatment revealed that this was also increased and was associated with increased plasma LH concentrations. However, after 1 year of treatment with anastrozole, all hormone concentrations measured were back within the control range. Mating behaviour was compromised in the treated rats, as indicated by a marked inability of the rats to produce copulatory plugs when placed with females. Mating could be restored in some animals by the administration of an oestrogen, thus demonstrating that anastrozole-treated rats were still fertile. Testis weight was increased in the majority of rats treated with anastrozole for 19 weeks and 1 year, with the exception of 10% of rats that had SCO testes. Excluding the latter, morphometric analysis did not reveal any obvious difference in germ cell number between control and anastrozole-treated animals. Histology of the testis, rete testis and efferent ducts appeared grossly normal in the majority of the rats and gave no obvious indication that testicular function was significantly altered by treatment with anastrozole.

Immature female rats received anastrozole via their drinking water to assess whether this was a suitable route

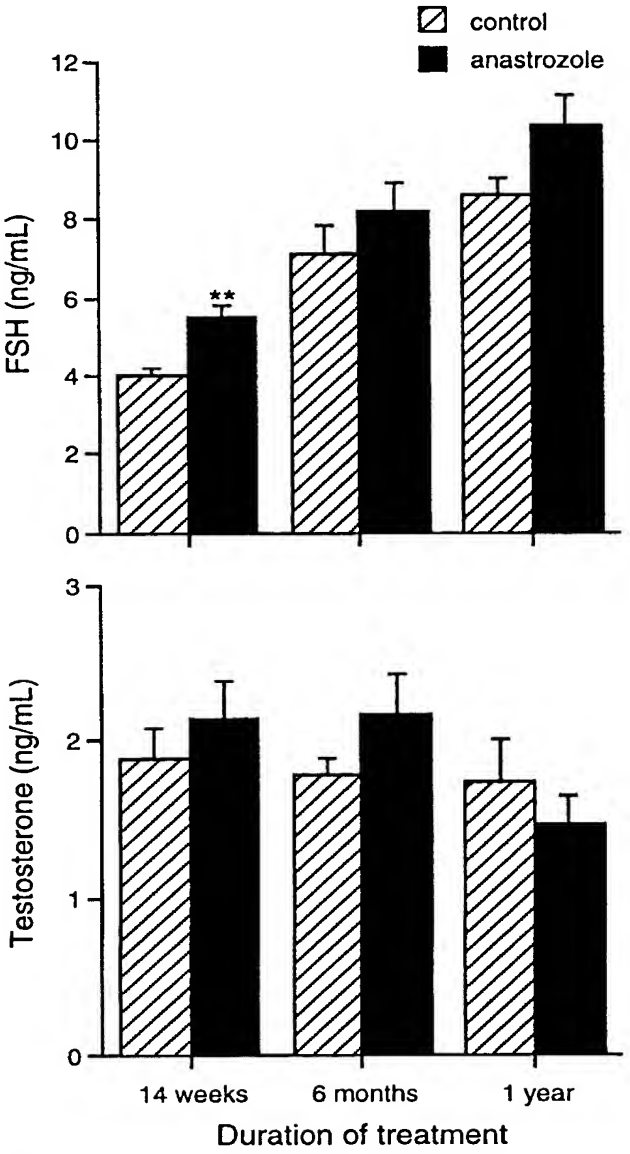

Figure 4 Effect of treatment of adult rats for up to 1 year with 200 mg/l anastrozole in drinking water on mean (± S.E.M.) plasma FSH (top) and testosterone (bottom) concentrations. Control group, $n=7–8$; anastrozole group, $n=8$. $^{**}P<0·01$ compared with control group.

of administration. The results obtained were generally in close agreement with the findings of other studies in adult female rats using another non-steroidal aromatase inhibitor, letrozole (Bhatnagar *et al.* 1993, Sinha *et al.* 1998). Administration of letrozole to adult cycling rats resulted in a dose-dependent decrease in uterine weight, an increase in gonadotrophin concentrations and a small increase in ovarian weight. Whereas one study reported a 75%

Table 5 Morphometric analysis of testis sections from adult rats treated with 200 mg/l anastrozole added to drinking water for 19 weeks, showing the percentage volumes of the main compartments of the testis, and total Sertoli cell and germ cell volumes (means ± S.E.M.)

	Treatment group	
	Control (n=4)	Anastrozole (n=5)
Volume per testis		
Interstitium (%)	2·62 ± 0·03	2·66 ± 0·18
Seminiferous epithelium (%)	79·4 ± 0·5	76·7 ± 3·9
Lumen (%)	17·8 ± 0·6	20·7 ± 4·0
Sertoli cell (μm^3/testis)	301 ± 7	362 ± 26
Total germ cell (μm^3/testis)	3900 ± 158	4080 ± 185
Total germ cell : Sertoli cell ratio	126 ± 0·3	115 ± 7

NB: SCO testes were excluded from the morphometric analysis.

suppression in serum oestradiol concentrations (Bhatnagar *et al.* 1993), the other could demonstrate only an 80% reduction in ovarian, but not plasma, oestradiol concentrations (Sinha *et al.* 1998). In our study, anastrozole induced only a 17% decrease in plasma oestradiol. There appears to be a problem with the accurate measurement of blood oestradiol concentrations in the rat, and the discrepancies just mentioned may be a reflection of the different assays used. At present, measurement of blood oestradiol in rats is therefore not an accurate index of the degree of aromatase inhibition *in vivo*. The difference in the degree of oestradiol suppression induced by letrozole in comparison with anastrozole is most probably a reflection of the shorter half-life of anastrozole *in vivo* (Dukes 1997). Both aromatase inhibitors suppress human placental aromatase activity *in vitro* to a similar extent, the IC_{50} for letrozole and anastrozole being 11·5 and 15 nM respectively (Bhatnagar *et al.* 1990, Dukes 1997). However, when tested *in vivo* using suppression of androstenedione-induced uterine hypertrophy as an index of aromatase inhibition, letrozole has a lower ED_{50} than anastrozole, 1–3 μg/kg compared with 0·5 mg/kg. The rapid clearance of anastrozole means that greater doses must be used to maintain the same concentration of aromatase inhibition. The results from our study in female rats demonstrated that anastrozole administered via the drinking water was bioactive *in vivo*.

Our results demonstrate clearly that, as in other species, the aromatisation of testosterone to oestradiol plays an important role in the regulation of FSH secretion in the rat. It is widely accepted that oestrogen can inhibit gonadotrophin secretion in men by acting at the pituitary gland (Finkelstein *et al.* 1991, Bagatell *et al.* 1994). This is reinforced by recent evidence that men with inactivating mutations in the genes for aromatase or ERα show increased serum FSH and LH concentrations (Smith *et al.* 1994, Morishima *et al.* 1995, Carani *et al.* 1997). Treatment of men with non-steroidal aromatase inhibitors also induces a rapid increase in FSH concentrations (Bhatnagar *et al.* 1992, Trunet *et al.* 1993, Hayes *et al.* 1999). Furthermore, other recent data would suggest that it is oestradiol alone, and not testosterone, which is responsible for negative feedback regulation of FSH in men (Hayes *et al.* 1999). The authors of that study concluded that both testosterone and oestradiol had a role in the negative feedback regulation of LH. This conclusion is consistent with the finding that suppression of aromatisation results in increases in LH concentrations in men, primates and dogs (Ellinwood *et al.* 1984, Juniewicz *et al.* 1988, Bhatnagar *et al.* 1992, Trunet *et al.* 1993, Plourde *et al.* 1994). The situation in the rat was believed to be different, as short-term administration of non-steroidal aromatase inhibitors failed to alter serum LH concentrations; FSH concentrations were not measured in either study (Bhatnagar *et al.* 1992, Plourde *et al.* 1994). However, oestrogens have been shown to regulate negatively the expression of the mRNA for all three gonadotrophin subunits in the male rat (Wierman *et al.* 1989). Our data show that there is differential regulation of FSH and LH secretion in the adult male rat, such that FSH is more sensitive to the effect of aromatase inhibitor treatment. The increase in FSH concentrations and the absence of a detectable change in oestradiol concentrations in blood in aromatase-treated males may indicate that aromatisation of testosterone locally within the hypothalamus/pituitary gland is responsible for this negative feedback loop.

Mutations resulting in oestrogen resistance or aromatase deficiency have yielded contradictory information on the relative importance of oestradiol in the regulation of testicular testosterone production (Eddy *et al.* 1996, Lindzey *et al.* 1998, Smith *et al.* 1994, Carani *et al.* 1997, Morishima *et al.* 1995, Fisher *et al.* 1998). Nevertheless, several studies involving the inhibition of aromatisation have shown a consistent and rapid increase in testosterone concentrations (~50%) in humans (Bhatnagar *et al.* 1992, Trunet *et al.* 1993, Hayes *et al.* 1999), primates (Ellinwood *et al.* 1984, Dukes *et al.* 1996, Shetty *et al.* 1998) and dogs (Juniewicz *et al.* 1988). In the present study, an increase

in plasma testosterone concentrations occurred within 1 week of anastrozole treatment and was maintained for approximately 5 months but not beyond this time. Other groups have been unable to demonstrate an effect on testosterone secretion in adult rats after administration of aromatase inhibitors, including anastrozole and fadrozole, for 1 or 2 weeks (Bhatnagar *et al.* 1992, Plourde *et al.* 1994), although these involved different routes of administration compared with the present studies. In our studies, anastrozole treatment of adult rats induced a 50% increase in testosterone concentrations, which is similar to that observed in dogs, primates and human, suggesting that the male rat is no different from these species in this respect.

Castration and testosterone replacement experiments in male rats have established that testosterone is necessary for sexual behaviour, and other studies using aromatase inhibitors or non-aromatisable androgens support the hypothesis that testosterone action is mediated in part by its aromatisation to oestradiol (Meisel & Sachs 1994). In our first study, we found that, after treatment with an aromatase inhibitor for 7–9 weeks, all but one of the treated rats failed to produce copulatory plugs when mated with a female. In the second study, administration of the synthetic oestrogen, DES, to rats treated with anastrozole before mating enabled some of the animals to produce copulatory plugs. ERKO mice also produce significantly fewer copulatory plugs in comparison with wild-type mice when mated, and show a marked inability to ejaculate (Eddy *et al.* 1996, Ogawa & Lubahn 1997), and it has been reported that ArKO male mice show decreased numbers of mounts in response to females (Honda *et al.* 1998). Furthermore, treatment of castrated rats containing testosterone implants with the aromatase inhibitor, fadrozole, for 2–4 weeks also reduced the frequency of ejaculations and intromissions (Bonsall *et al.* 1992). Our results would support other studies demonstrating a requirement for oestrogen action in the brain for mating.

Testis weight was significantly increased by chronic treatment with anastrozole, although, for the majority of the animals, no impairment of spermatogenesis was observed. Occasional (<2%) abnormal seminiferous tubules were detected after treatment for 10 or 19 weeks and 1 year but, in general, spermatogenesis remained grossly normal in the majority of treated rats. In the course of experiment 2, five out of a total of 55 anastrozole-treated rats were identified to have SCO testes (unilateral or bilateral). Such testes increase in incidence in ageing rats, although this does not provide an obvious explanation for our findings. In view of the role now established for oestrogens in the function (fluid resorption) of the efferent ducts and the consequences of its disruption in ERKO mice (Hess *et al.* 1997*a*), the possibility remains that the sporadic cases of unilateral or bilateral SCO testes in anastrozole-treated rats are related to altered fluid dynamics. If this is the case, it must mean that anastrozole treatment exerts only borderline effects on oestrogen concentrations in the efferent ducts.

Fertility was not compromised by anastrozole treatment. Treatment of pubertal male rats with fadrozole for 60 days followed by a recovery period of 30 days was found to have no effect on subsequent fertility (Nunez *et al.* 1996). Other studies involving the chronic inhibition of aromatisation in rats, dogs or primates have given conflicting results. Administration of letrozole to adult male rats for 6 months was reported to have no effect on the testes, but treatment of dogs for 3 months resulted in Leydig cell hyperplasia/hypertrophy and arrest of spermatogenesis (Junker Walker & Nogues 1994). This is in contrast to treatment of dogs with fadrozole for 6 months, which had no effect on sperm counts or gross testicular morphology (Juniewicz *et al.* 1988). Long-term treatment of adult bonnet monkeys with an analogue of letrozole resulted in a 90% reduction in sperm production after treatment for 55 days; flow cytometric analysis demonstrated a reduction in round and elongating spermatids, implying a defect in spermiogenesis (Shetty *et al.* 1998). Administration to adult male rats of a protein purified from the ovary that had aromatase inhibitory activity also induced a reduction in the number of elongate spermatids (Tsutsumi *et al.* 1987), and a similar late-onset lesion in ArKO mice has recently been described (Robertson *et al.* 1999). The latter study found that a defect occurs in differentiation of round into elongate spermatids, resulting in fewer sperm and subsequent infertility. The testicular phenotype of ArKO mice shows marked differences from that seen in ERKO mice, in which the impairment to spermatogenesis is believed to be the result of fluid accumulation in the seminiferous tubules induced by a reduction in fluid resorption by the efferent ducts (Eddy *et al.* 1996, Hess *et al.* 1997*a*). The available data would therefore imply a role for oestrogen in the regulation of spermatogenesis but our studies of anastrozole-treated rats were unable to confirm this (see below).

Despite the fact that we could demonstrate that plasma FSH concentrations were significantly increased, and that normal mating behaviour was inhibited in anastrozole-exposed male rats – both good indirect indicators of inhibition of aromatisation – we were unable to detect a significant decrease in plasma oestradiol concentrations; however, our radioimmunoassay did detect a modest but significant decrease (~20%) in oestradiol concentrations measured in testicular interstitial fluid. This obviously raises doubt as to the degree of inhibition of aromatisation in our animals. However, we believe that the lack of availability of sensitive and specific oestradiol RIAs for the rat is part of the problem. Other investigators have reported similar difficulties in assessing the degree of suppression of aromatisation in rats by measurement of blood concentrations of oestradiol (Sinha *et al.* 1998, M Dukes personal communication). Indeed, Sinha *et al.* (1998) were unable to detect a significant effect of

ovariectomy on serum oestradiol concentrations, using a sensitive RIA and a yeast-based bioassay. Another difficulty in the evaluation of the effects of anastrozole in the male rat is the fact that this compound has a relatively short clearance half-life, ~2 h (Dukes 1997), which is in contrast to that found in female rats (6 h), monkeys (7 h), dogs (14 h) and women (48 h) (Dukes *et al.* 1996). We tried to overcome this limitation by administering a relatively high dose of anastrozole to rats via their drinking water, to increase the exposure period. The fact that we were able to demonstrate a rapid and consistent effect of anastrozole treatment on pituitary function and mating behaviour implies that we were successful in achieving adequate suppression of aromatisation in the brain. However, the lack of effects on testicular function, although consistent with the results from other studies in male rats using aromatase inhibitors (some of which have long half-lives), are not consistent with findings obtained from ERKO or ArKO mice. Although there is no ready explanation of this inconsistency, there are some obvious possibilities. First, it is possible that the aromatase inhibitors do not gain ready access to sites of aromatase expression in the testis such as the germ cells (Janulis *et al.* 1998). Second, the increase in testosterone concentrations induced by anastrozole treatment may provide increased concentrations of substrate for aromatisation or may induce increased expression of aromatase (Roselli & Resko 1997). Third, the presence of soy-derived phytoestrogens in the diet of the rats may compensate for any local reduction in oestrogen concentrations resulting from inhibition of aromatase activity, as has been reported for ArKO mice (Simpson *et al.* 1999). Fourth, spermatogenesis in the rat may have a different degree of dependence on oestrogens than does that in the mouse. These possibilities should be taken into account in the design of future studies seeking to manipulate endogenous oestrogen concentrations in the male rat. The aim of these studies was to create a model of oestrogen insufficiency, but the effects observed suggest that aromatase has been adequately suppressed only in the brain.

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